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MARINE ALGAL ASSAY PROCEDURE: BOTTLE TEST

Pacific Northwest Environmental Research Laboratory
Corvallis, Oregon

December 1974

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16. ABSTRACT

Describes protocol for a standardized primary producer nutrient bioassay for assessment of the effect of cultural eutrophication in estuarine and coastal marine areas. It is a companion procedure to the freshwater ALGAL ASSAY PROCEDURE:BOTTLE TEST, EPA, August, 1971.

The green biflagellate unicellular alga Dunaliella tertiolecta Butcher (DUN clone) was selected as the bioassay organism because of its wide salinity tolerance, sensitivity to incremental additions and natural levels of critical or limiting nutrients, excellent replication and simple evaluation characteristics. Contains numerous references.

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NATIONAL ENVIRONMENTAL RESEARCH CENTER
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FOREWORD

The success and widespread acceptance of the freshwater Algal Assay Procedure: Bottle Test, has prompted the Eutrophication and Lake Restoration Branch to develop a companion procedure of the test to deal with problems of assessment of cultural eutrophication in estuarine and marine coastal situations.

The developmental work at the Pacific Northwest Environmental Research Laboratory, Corvallis, Oregon, consisted of choosing suitable algal species, investigations to provide necessary background physiological data, and field testing the process. It was necessary to find algal species that had wide salinity tolerance, low or predictable nutrient carry over, simple evaluation characteristics, good replicability, and predictable growth response to various nutrient concentrations.

This work has been under the immediate direction of William E. Miller and David T. Specht. To them goes much credit for effectively developing the procedure.

Thomas E. Maloney, Chief
Eutrophication and Lake Restoration Branch

MARINE ALGAL ASSAY PROCEDURE: BOTTLE TEST

1. Introduction

In February 1969, the Joint Industry/Government Task Force on Eutrophication published the Provisional Algal Assay Procedure (PAAP). Shortly after publication of the PAAP, a group consisting of government, university and industrial laboratories undertook a comprehensive research program to improve and evaluate it. While the PAAP consisted of three fundamental test procedures, the first phase consisted of refining the Bottle Test. After more than two years of intensive research and development, the Algal Assay Procedure: Bottle Test was published in August 1971.

During the course of evaluating the Bottle Test, an inter-laboratory precision test was carried out by eight laboratories. The results of this was published in October 1971 and entitled "Inter-Laboratory Precision Test."

The Algal Assay Procedure Bottle Test has received widespread acceptance and it was readily recognized that a similar test should be developed to study eutrophication problems in estuarine and marine coastal waters. Although the laboratory procedure is ready for routine use, as was the case with fresh-water test, further evaluation relating to specific field situations will be necessary (Specht and Miller, In Press; Specht, In Press).

2. Principle

The principle of this marine version of the Algal Assay Procedure is essentially the same as previously published, but will be outlined here.

The assay is based on Liebig's Law of the Minimum, modified by rate considerations [see O'Brien (1972); Holmes (1973); Kelley and Hornberger (1973); O'Brien (1973)] in which the biomass produced in a given amount of time (rate) is related to the available concentration of the limiting nutrient.

The assay has been designed to assess receiving waters of varying salinity as to nutrient status, biostimulation potential, and sensitivity to change in nutrient concentration.

The test is intended to identify algal growth-limiting nutrients, biologically determine their availability, and quantify the biological response to changes in concentration. It is anticipated that development work in the future will show the various inhibitory effects of certain pollutants on algal growth potential.

These measurements are made in a uniform manner by inoculating a batch of test water with a selected test alga and determining algal growth according to an established test protocol.

2.1 The maximum specific growth rate is related to the concentration of the rate limiting nutrient present. The maximum standing crop is proportional to the initial amount of the limiting nutrient available.

2.2 All comparative growth responses should be statistically analyzed and significant levels of differences reported. For most purposes a 95 percent significance level can be considered statistically significant.

3. Planning and Evaluation

The specific experimental design of each algal assay must be dictated by the actual situation. It is extremely important that all pertinent ecological factors be considered in planning a given assay to insure that valid results and conclusions are obtained. As a minimum, the following specifics must be considered by each investigator who plans to conduct algal assays for the purposes listed above.

3.1 Selection of test waters - Water quality of estuaries will vary greatly with time and location. Sampling programs should be established so that meaningful data will be obtained.

3.11 Spatial variations - The influence of point sources of nutrients or pollutant input on algal biomass production or growth rate may be determined by sampling upstream and downstream from the point source, taking tidal fluctuations into account.

3.12 Temporal variations - Water quality in an estuary will vary not only with season, but with each tide. Sampling schedules should be arranged to take into account this variation, sampling preferably at high water, or at both high water and the

following low water. An effort should be made to determine whether the estuary is stratified or well mixed and sampling schemes should be modified to account for this potential variable.

3.2 Determination of limiting nutrients - Any of the essential nutrients may be limiting to algal growth. Bioassays are limited to examination of only a few nutrients which are likely to be limiting. See Appendix 3 (in AAP, August 1971) for an example of an experimental plan.

4. Apparatus

4.1 Sampling and Sample Preparation

4.11 Water Sampler - Non-metallic

4.12 Sample Containers - Sterilizable (borosilicate glass, linear polyethylene, polycarbonate, or polypropylene, for autoclaving); polyethylene "cubitainers" sterilizable by acid washing.

4.13 Membrane filter apparatus - 123 mm filter transfer pump, for use with .45μm porosity prewashed filters, or 47 mm standard apparatus.

4.14 Autoclaving - Considered at this time to be too drastic a treatment because of precipitation of salts.

4.2 Culture and Incubation

4.21 Culture vessels. Erlenmeyer flasks of good quality borosilicate glass such as Pyrex^{R} or Kimax^{R}. The same brand of glass

should be used within a given laboratory. When trace nutrients are being studied, special materials, such as Vycor^{R}, polycarbonate, or coated glassware should be used.

For gas exchange considerations, a contact surface to volume ratio should be used as follows:

40 ml in 125 ml flask

60 ml in 250 ml flask

100 ml in 500 ml flask

4.22 Culture closures - Culture flask closures are preferably polyurethane foam plugs (Gaymar^{R}), or can be loose fitting aluminum foil or small inverted beakers. These must be tested for toxic effects on algal growth.

4.23 A constant temperature room, or equivalent incubator is needed to provide temperature control at 18° $\pm$ 2°C.

4.24 Illumination - "cool white" fluorescent lamps to give at least 250 ft-c (2152 lux), preferably 400 ft-c (4304 lux). Adjust lighting to give $\pm$ 10 percent illumination over the entire shaker platform. Measure adjacent to liquid level in flask.

Note: The energy level output of a bank of six 48-inch "cool white" fluorescent lamps (GE 40 watt, @ 60 Hz) was approximately 1300 $\mu w/cm^2$ (range, 380-760 nm) at a distance of 26 3/4 inches, as measured with an ISCO Model SRC spectroradiometer. Using the same measurement

geometry, a Weston Model 756 Illumination Meter read 400 ft-c. All reflecting surfaces were matte white.

Therefore, utilizing a calibrated illumination meter with a foot-candle readout, one may, by adjusting the height of the lights, achieve a known energy level output of 1300 $\mu w/cm^2$.

For further discussion of the problems of the differences in absorption of light by photosynthesizing organisms and by man's eye and their measurement, see Tyler (1973).

4.25 Light meter - Must be calibrated against a standard light source or meter.

4.3 General Apparatus

4.31 Analytical balance, capable of weighing 100 gm with a precision of $\pm$ 0.1 mg.

4.32 Microscope - Good quality general purpose microscope with illuminator.

4.33 Haemocytometer or plankton counting slide.

4.34 pH meter - Scale of 0-14 units with accuracy of $\pm$ 0.1 pH unit.

4.35 Oven, dry heat, capable of temperatures to 120°C.

4.36 Centrifuge - Capable of centrifugal force of at least 1000 x g.

4.37 Spectrophotometer or colorimeter - For use at 600-750 nm.

4.4 Optional, but desirable equipment.

4.41 Electronic cell counter.

4.42 Fluorometer.

4.43 Shaker table, capable of 110 oscillations per minute.

5. Sample Collection, Transport, Preparation and Storage

5.1 Collection - Use non-metallic water sampler and sterilizable containers (see Section 4.11, 4.12). Containers should be pre-rinsed with a portion of the sample to acclimate the interior surface to the nutrient concentrations of the sample. Containers suspected of toxic or nutrient contamination should not be used.

5.2 Transport condition - Fill containers to leave minimum air space, refrigerate (ice) and keep in dark during transporation.

5.3 Preparation - Mix sample thoroughly and remove enough sample for pre-filtration chemical analysis (if desired), and filter to remove indigenous algae. Autoclaving is not recommended for marine or estuarine samples at this time.

5.31 Membrane filtration - Removes indigenous algae and detritus. Bioassay then determines growth limiting nutrient not taken up by indigenous organisms removed by filtration. Pretreat 0.45 μm membrane filter by passing at least 50 ml double glass distilled water through it (47 mm filter) or one liter of water (123 mm filter). Discard this filtrate. Proceed to filter the quantity of sample needed under reduced pressure of 0.5 atmosphere or under pressure of less than 1.5 atmosphere.

If a great deal of suspended matter is present, a prewashed prefilter may be used, or precentrifugation utilized.

5.4 Storage - Samples should be stored at 0-4°C in the dark, excluding air bubbles in the container. If prolonged storage is anticipated, samples should be prepared (filtered) first.

6. Synthetic Algal Nutrient Medium

6.1 Basal Medium: Modified Burkholder's* Artificial Seawater (ASW) with NAAM levels of the following nutrients: N, P, Fe, and Na_2EDTA. Use Analytical Reagent or Reagent Grade chemicals.

Compound	grams/l	grams/4 l
NaCl	23.48	93.92
Na_2SO_4	3.92	15.68
$NaHCO_3$	0.19	0.76
KCl	0.66	2.64
KBr	0.10	0.38
H_3BO_3	0.03	0.10
$MgCl_2 \cdot 6H_2O$	10.61	42.44
$SrCl_2 \cdot 6H_2O$	0.04	0.16
$CaCl_2 \cdot 2H_2O$	1.47	5.88
H_2O to	1,000 ml	4,000 ml

filter through prewashed 0.45 µm membrane filter.

*Ref. Burkholder, P. 1963. Some nutritional relationships among microbes of the sea sediments and waters. In: Symposium on Marine Microbiology, Ed. C. H. Oppenheimer. pp. 1133-1150. Thomas, Springfield, Illinois.

FOR DILUTION TO VARIOUS SALINITIES: (4 liter batches)

Salinity °/₀₀	ASW Stock, l	H_2O (glass distilled), l
35	4.000	0.000
30	3.43	0.57
24	2.74	1.26
20	2.29	1.71
16	1.83	2.17
12	1.37	2.63
8	0.91	3.09
5	0.57	3.43

For any given final salinity, mix well, adding the following NAAM levels of nutrients:

$NaNO_3$	102 mg/4-l batch (4.2 mg N/l)
K_2HPO_4	4.18 mg/4-l batch (0.186 mg P/l)
Na_2EDTA	1200 µg/4-l batch (300 µg/l)
*NAAM trace metal mix (minus $FeCl_3$)	

Filter through 0.45 µm membrane filter, add AFTER filtration, sterilized $FeCl_3$, 384 µg/4-l batch (33.05 µg Fe/l).

*Add the following: 0.0928 g H_3BO_3; 0.208 g $MnCl_2$ $4H_2O$; 0.016 g $ZnCl_2$; 0.714 mg $CoCl_2 \cdot 6H_2O$; 0.0107 mg $CuCl_2 \cdot 2H_2O$; 3.63 mg $Na_2MoO_4 \cdot 2H_2O$; make up to 500 ml., adding 1 ml of this concentrate to each liter of media.

Adjust to pH of 8.0 $\pm$ 0.1, if necessary.

6.2 Stock solutions - Stock solutions of some salts may be made up 1000 times the final concentration. Practically speaking, these are N, P, Fe, EDTA, trace metals, Sr, Br. The remaining salts are required in amounts that are impractical to hold in stock solutions.

6.3 Reference medium - Make up ASW to 20 °/₀₀, store in dark at 4°C excluding air from the container. This concentration should be used to raise the inoculum. The reference medium should be diluted from 35 °/₀₀ stock, according to the dilution schedule, to match the salinity of the sample.

7. Inoculum

7.1 Test Algae

7.11 Dunaliella tertiolecta Butcher (DUN clone).

7.12 Thalassiosira pseudonana Hasle and Heimdal,* (CN clone). (Cyclotella nana Hustedt)

*This organism is currently being evaluated for use. If used, add Si and vitamins to stock medium:

0.6 mg $Na_2SiO_3 \cdot 9H_2O$/l

1.0 ml vitamin mix/l

7.121 Preparation of vitamin mix: Stock - 0.1 mg/ml Biotin (dissolve by warming, if necessary). 0.1 mg/ml B_{12}. Make 10 ml in double glass distilled water, keep frozen and sterile.

Take 1 ml vitamin mix, add 20 mg thiamine hydrochloride to 100 ml double glass distilled water. Dispense into 1 ml ampoules (or sealable containers). Autoclave 5 minutes, store frozen. Use 1 ml/l final solution. (From Curl, H. W. 1971). Preparation of Basic Culture Media. Mimeograph copy. Oregon State University Dept. Oceanography.

7.2 Source of test algae - Available from the Eutrophication and Lake Restoration Branch, Pacific Northwest Environmental Research Laboratory, NERC-Corvallis, EPA, 200 SW 35th Street, Corvallis, OR 97330.

7.3 Maintenance of stock cultures.

7.31 Media. (See Section 7 and 8.12)

7.32 Incubation conditions. (See Section 4.24) 18° ± 2°C under continuous illumination of 400 ft-c (4304 lux ± 10 percent for Dunaliella).

18° ± 2°C under continuous illumination 550-600 ft-c (5900-6500 lux) ± 10 percent for Thalassiosira.

7.33 Transfer of cultures - Transfer under aseptic conditions at least every seven days from previously unopened stock flask, 1 ml into 100 ml new media.

7.34 Age of Inoculum: Use cultures 5-7 days of age, preferably on the younger side so cells are in or near log phase of growth.

7.4 Preparation of Inoculum - Cells from stock culture should be centrifuged in sterile centrifuge tubes and the supernatant discarded.

The sedimented cells should be resuspended in filter sterilized 20 °/₀₀ ASW less N, P, EDTA, trace metals and Fe, and again centrifuged. Discard the supernatant and again resuspend the pellet in the 20 °/₀₀ ASW as before, diluting to the appropriate concentration for the inoculum. (see Appendix 7.5 for example with typical calculations.)

7.5 Strength of inoculum - The prepared cell suspension (Appendix 7.5) should be counted and adjusted by dilution to 10,000 cells/ml so that a 1 ml inoculum iinto 99 ml of sample + spikes will give a final concentration of 100 cells per ml. (Approximately 0.02 - 0.03 mg/l dry weight, see Appendix 7.5).

8. Test Conditions

8.1 Temperature - 18° - 20°C

8.2 Illumination - Continuous "cool white" fluorescent lighting 400 ft-c (4304 lux) ± 10 percent for Dunaliella; 550-600 ft-c (5900-6500 lux) ± 10 percent for Thalassiosira (see Section 4.24).

8.3 Shakers - To facilitate gas exchange, Dunaliella should either be continuously shaken at 110 oscillations/minute or kept static and the flasks swirled by hand twice/day (continuous shaking is desirable for uniformity). Thalassiosira should not be shaken, but kept static and swirled once/day by hand (i.e., as when mixing prior to sampling).

9. Procedure

9.1 Preparation of Glassware - All glassware associated with

the test should be washed with phosphate-free detergent or sodium carbonate and rinsed thoroughly with tap water. This is followed by a rinse with 10 percent by volume of reagent grade hydrochloric acid (HCl); glassware is filled momentarily with the HCl solution, swirled, dumped into the next flask, filled with a 10 percent by weight reagent grade sodium carbonate (Na_2CO_3) to neutralize the glass surface, then rinsed 5 times with deionized water, oven dried, inverted on racks at 105°C, capped with aluminum foil or stoppered with foam plugs (Gaymar^R) and autoclaved at 15 psi for 15 minutes.

Before dispensing, flasks should be prerinsed with an aliquot of the medium or test water to be used, to "acclimate" the glass surfaces to the concentrations of metals and nutrients in these waters.

9.2 pH Control - Sea water is usually a good buffer, and pH problems should not occur except perhaps with the very low salinity estuarine waters (below 5 °/$_{oo}$). Control of pH is accomplished by using optimal surface to volume ratios and adequate mixing to allow availability of carbon dioxide.

9.3. Growth Parameters - Two parameters are used to describe the growth of a test alga in the Bottle Test: maximum specific growth rate and maximum standing crop. Either or both of these parameters may be determined, depending on the objectives of the particular assay.

10. Spikes

The quantity of cells that may be produced in a given medium is

limited by the substance that is present in the lowest relative quantity with respect to the needs of the organism. If a quantity of the limiting substance were added to the medium, cell production would increase until this additional supply was either depleted or until some other substance became limiting to the organism (see Appendix 10). Additions of substances other than that which is limiting would yield no increase in cell production. Nutrient additions may be made singly or in combination, including wastewaters, and the growth response may be compared to unspiked controls to identify those substances which limit growth rate or cell production. The selection of spikes, e.g., nitrogen, phosphorus, iron, sewage effluents, etc., will depend on the answer being sought.

In all instances, the volume of the spike should be as small as possible. The concentration of spikes will vary and must be matched to the waters being tested. Two considerations should be taken into account when selecting the concentration of spikes: (1) the concentration should be kept small to minimize alterations of the sample, but at the same time it should be sufficiently large to yield a potentially measurable response; and (2) the concentration of spikes should be related to the fertility of the sample. To assess the effect of nutrient additions, they must be compared to an unspiked control of the test water. If the control is quite fertile, cell production will be high and flask-to-flask variations in the controls might mask the effect of small additions of the limiting nutrient (see Appendix 10).

10.1 Auxiliary Spikes - In addition to spikes for the purpose of determining stimulatory or inhibitory effects on algal growth in test waters, it is sometimes necessary to check for the possibility that the test water contains some toxic material which could influence results. To check for toxic materials, the test water may be spiked with the elements in complete synthetic medium (see Section 6). If no increase in growth occurs, the presence of toxic materials is suspected. In some situations, dilution of the sample or the addition of a chelator will eliminate toxic effects.

11. Data Analysis and Interpretation (See Algal Assay Procedure: Bottle Test, August, 1971, pp. 21-23)

11.1 Introduction - The fundamental measure used in the Algal Assay: Bottle Test to describe algal growth is the amount of suspended solids (dry weight) produced and this is determined gravimetrically. Other biomass indicators such as those shown in 9.6 may be used; however, all results presented must include experimentally determined conversion factors between the indicator used and the dry weight of suspended solids.

Several different biomass indicators should be used whenever possible because biomass indicators may respond differently to any given nutrient limiting condition.

11.2 Reference Curves - Results of spiking assays should be presented together with the results from two types of reference samples; the assay reference medium and unspiked samples of the

water under consideration. Preferably the entire growth curves should be presented for each of the two types of reference samples. The results of individual assays should be presented in the form of the maximum specific growth rate (with time of occurrence) and maximum standing crop (with time at which it was reached). (see AAP, August 1971).

A P P E N D I C E S *

*Each Appendix is numbered to correspond with that Section to which its contents are related. Some Sections do not have a corresponding Appendix.

APPENDIX 4

4.14 Autoclaving - Several alternatives to autoclaving are being investigated to avoid the irreversible precipitation problems caused by heat input. The most promising method is that of explosive decompression. This is accomplished by loading the sample into a large (1 gallon capacity) chemical "bomb," charging with dry nitrogen (N_2) at pressures not to exceed 2,000 lbs/in^2, and allowing the contents to come to equilibrium. The contents of the bomb are released under controlled procedures into a container. Under ambient pressure, the cellular material contained therein releases the absorbed nitrogen by rupturing cell walls, effectively sterilizing the sample with respect to algae. Some bacterial and yeast spores are capable of withstanding such treatment, but no algae have been reported as surviving. No available nitrogen is contributed to the sample, there is no heat input (in fact, upon release, the sample undergoes adiabatic expansion), and the discharged sample may be kept under a blanket of inert nitrogen.

4.22 Flask Closures - Closures must demonstrate the following properties:

maintain integrity of culture

survive autoclaving

provide uniform gas exchange

be non-toxic to the test species

While several types of closures have proven to be toxic, primarily by volatiles released during autoclaving, some specific types have proven satisfactory:

aluminum foil

Delong^{R} closures (stainless steel)

Gaymar^{R} polyurethane foam plugs

The metal closures have the disadvantage of blocking or reflecting light. Other types or brands of plastic foam plugs must be tested for toxicity.

APPENDIX 7

7.11 Taxonomy:

Dunaliella tertiolecta Butcher (DUN clone) is a green unicellular flagellate, Class Chlorophyceae, Order Volvocales, Family Polyblepharidaceae. Cells are ovoid, 5-8 x 10-12 µm, with two long flagella at the anterior end and have one cup shaped chloroplast, with a single pyrenoid, at the posterior end.

Asexual reproduction takes place by longitudinal division, although the alga may occasionally reproduce sexually, by isogamy, producing up to 16 zoospores. Under growth stimulating conditions, asexual reproduction prevails.

Thalassiosira pseudonana Hasle and Heimdal (CN clone) Cyclotella nana Hustedt is a centric diatom, Class Bacillariophyceae, Order Centrales, Family Discoideae, 4-9 µm in diameter. The usual reproductive process is asexual fission, but sexual fusion by anisogamy may occur. Although succussive vegetative division may tend to reduce cell size, periodic auxospore formation will restore cell size.

7.4 Preparation of Inoculum - Cells are obtained from a stock culture, preferably in log phase of growth. Take, for instance, 40 ml of stock culture, pour into sterile centrifuge tube, cap, and centrifuge for 10 minutes at 2,000 rpm. Decant the supernatant, refill to mark with filter (0.45 µm) sterilized 20 °/₀₀ ASW less N, P, Fe, EDTA, and

micronutrients (make up and keep in separate container for this purpose), and centrifuge again for 10 minutes at 2,000 rpm. Again decant the supernatant, and refill with 20 °/°° ASW diluent. Take a 1 ml subsample of this preparation and count.

For example: Counting with an electronic particle counter, at a dilution of 1 ml sample to 99 ml of 1% NaCl electroyte (1:100 dilution), the machine counts the number of cells in 0.5 ml of the 1:100 dilution:

Count	MCV (μm^3)	Machine settings: Amplification 1/½ Aperture: 1/½
6217	81	
6182	78	Background count in filtered saline electrolyte - 10 -
6068	78	
6074	77	
6039	76	
	390/5 = 78 ± 1.9	

Enter these data into the following data reduction equation:

$$\frac{([a-x]\ mcv_a) + ([b-x]\ mcv_b) + \ldots ([n-x]\ mcv_n)}{n}$$

$$\cdot(1+[2.52 \times 10^{-6}\{\frac{([a-x]\ mcv_a) + ([b-x]\ mcv_b) + \ldots ([n-x]\ mcv_n)}{n}\}])$$

$$\cdot Y \cdot \text{(dry weight factor)} = \text{calculated dry weight}$$

where: a, b,...n = numerical cell counts

$mcv_a, mcv_b, \ldots mcv_n$ = associated mean cell volumes in μm^3

$(1+[2.52 \times 10^{-6}])$ = coincidence correction factor

Y = dilution factor (multiplier)

X = background count

(dry weight factor) = 8.66×10^{-7} mg/μm^3 cell volume, for Dunaliella tertiolecta;approximately the same for Thalassiosira pseudonana.

(The second part of the expression is a coincidence correction factor to account for the statistical probability of more than one cell passing through the orifice at a given time and appearing electronically as one cell twice as large as it really is. 2.52×10^{-6} is the factor for a 100µm orifice; other orifice sizes require different numbers, which can be supplied by the manufacturer of the counter.)

This expression, which may be set up on a small programmable desk calculator, can be arranged to yield the corrected cell count and the calculated dry weight.

Corrected count for this example: 1,240,000 cells/ml in stock culture or 83.8 mg/l calculated dry weight.

Needed: 100 cells/ml; 100 mls/flask; and, for example, 100 flasks or 10,000 cells/flask x 100 flasks to total 1,000,000 cells in 100 ml of inoculum.

Since there are 1,240,000 cells/ml in the stock preparation:

$$\frac{1,000,000}{1,240,000} = 0.806 \text{ ml of stock preparation}$$

Add 0.81 ml of stock to a 100 ml volumetric flask, and top up with 20 °/₀₀ ASW diluent. After thorough shaking to mix, withdraw a 1 ml sample and count.

Since this is a more dilute cell suspension, dilute only 1 + 9 (1:10).

Count	MCV (μ^3)	Background - 10 -
655	82	
642	82	
585	72	
620	71	
659	70	
	75 ± 6.1	

= 12,500 cells/ml

This amounts to a final flask concentration of 125 cells/ml, which is about 20 °/₀ too high. To correct, calculate:

12,500 x 99 (mls inoculum left in flask)

1,238,000 (Total number of cells left in flask)

1,000,000 (Number of cells desired)

214,000/12,300 (Excess cells/ml concentration)

= 19.00 ml (amount to remove from inoculum flask)

Remove 19.00 ml from the inoculum flask and top up with the 20 °/₀₀ ASW diluent, withdraw another 1 ml sample, dilute 1:10 and count:

Count	MCV (μm^3)
527	75
536	73
526	76
499	69
506	70
= 10,189 cells/ml	70 ± 3.0

The final flask concentration at inoculation is now 102 cells/ml or, 0.064 mg dry weight/liter.

The proper concentration may be obtained after the first dilution and no other manipulations are necessary. This, however, is usually not the case.

7.5 Strength of inoculum - Because nutrient carryover in an inoculum could prejudice results of the bioassay in more austere water samples, the initial inoculum level was reduced from 1,000 cells/ml (0.3 mg/l dry weight) to 100 cells/ml (0.03 mg/l dry weight). Although this introduces a detectable lag in growth response, growth rate appeared unaffected and the final yield was identical at day 10 or 12 (see Figure 1). The inoculum was dispensed in a 1 ml volume.

APPENDIX 10

10.1 Laboratory and Field Sample Data

The biomass response of the test alga (Dunaliella) at day 10 is graphically illustrated in Figures 2, 3, and 4 in the form of a 3-dimensional response-surface. These responses indicate that, in ASW, Dunaliella will show significant growth response at 2.5 to 50 µg/l phosphorus (Figure 5), 10.0 to 1,000 µg/l nitrate nitrogen (Figure 6), and 10.0 to 1,000 µg/l ammonia nitrogen (Figure 7), in 5 °/₀₀ to 35 °/₀₀ salinity.

In ASW, 5 °/₀₀ to 35 °/₀₀ salinity, Dunaliella produces an average of 1.08 mg dry weight per µg of phosphorus, 0.03 mg dry weight per µg of nitrate nitrogen and 0.08 mg dry weight per µg of ammonia nitrogen (see Table 1).

Dunaliella is consistent in its response, showing excellent replication. All experimental runs were performed in triplicate. An example of this consistency can be illustrated in the result of an investigation of nitrilo-tri-acetic acid, (NTA), as a potential nitrogen source in natural water samples. In addition to control, four levels of NTA, 0.05 to 1.0 mg/l (expressed as N), were added to water samples from six Oregon estuaries (five of which were nitrogen limited at the time) in triplicate. NTA neither stimulated nor inhibited growth in any sample in the time period; a t-test (13 degrees of freedom) showed no significant difference between an increase in NTA-N and dry weight produced. The normalized standard deviation for the entire run was less than ± 15 percent.

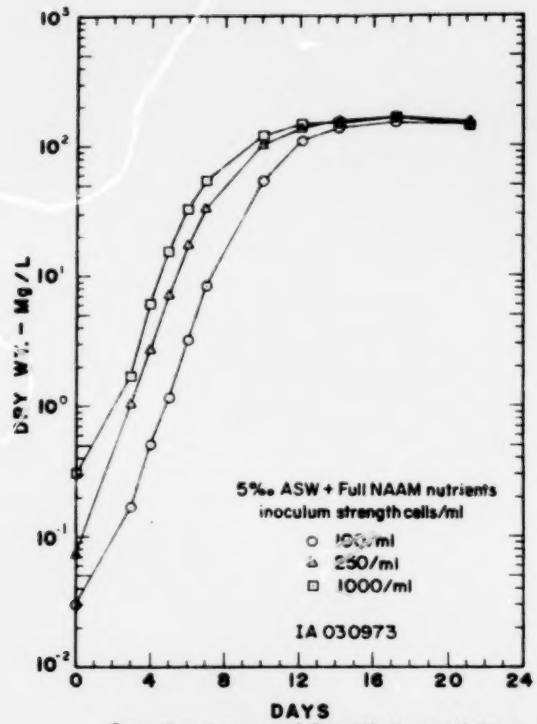
APPENDIX 11

11.4 Identification of growth-limiting nutrient.

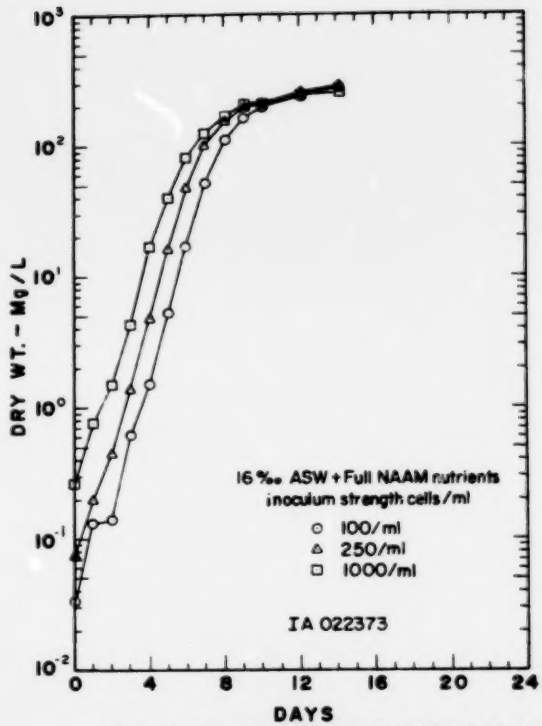
A set of samples taken July 25, 1973, from five southern Oregon Coastal estuaries (Figure 8) at high water and assayed with Dunaliella showed that all except the Umpqua River were growth limited by nitrogen. The Umpqua River station (11.6 °/₀₀ salinity) responded significantly to phosphorus spikes, but not to nitrogen (see Figures 9 and 10) (Specht and Miller, In Press).

In nitrogen-limiting situations, the growth rate of the test algae is significantly greater when ammonia nitrogen is added than when nitrate nitrogen is added. However, if allowed to incubate for a sufficient period of time, there is no significant difference between the final dry weight yields obtained with either of the nitrogen sources.

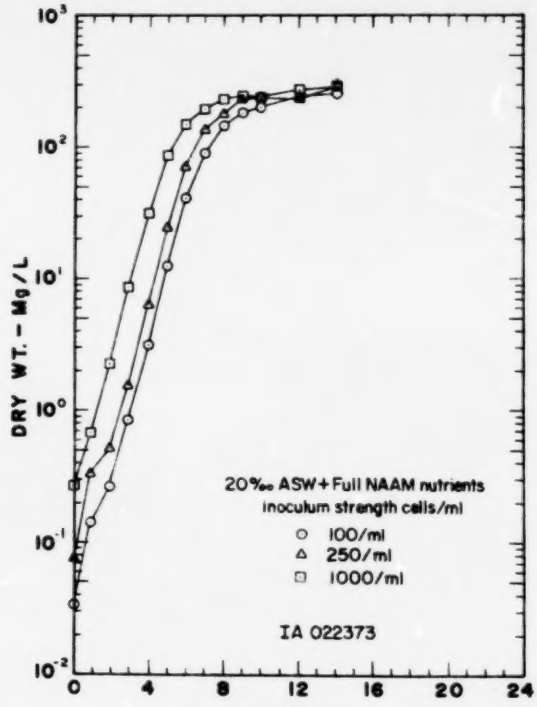
Assays performed on a set of samples taken on the low and following high tides in the Yaquina Bay estuary, February 9, 1974, illustrates the ability of the assay to define the boundary between nitrogen-limited seaward water and phosphorus-limited landward water as it moves in and out of the bay. Table 2 (Specht, In Press) shows the control dry weights, growth-limiting nutrient, salinity, phosphorus and nitrogen levels, and the dry weight produced per unit of the limiting nutrient as compared with the biomass produced in ASW (adjusted for salinity; see appendix 10.1, paragraph 2). It is interesting to note the degree of dependence and linearity of growth upon the limiting nutrient concentration, rather than upon salinity.



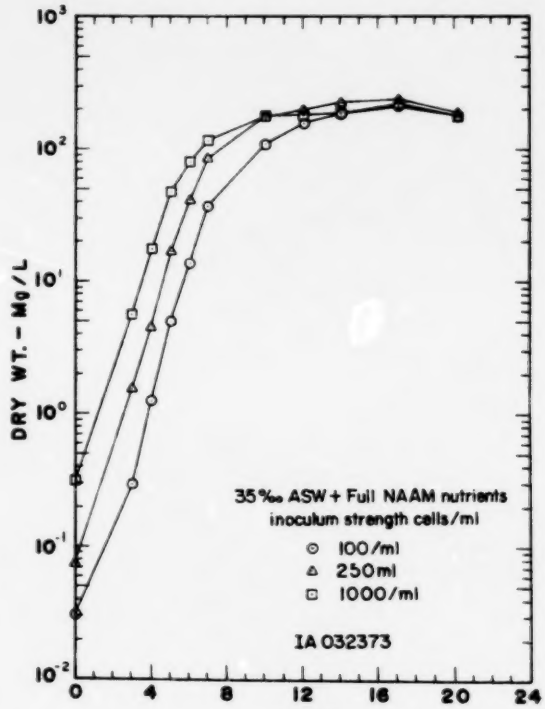
Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta

Fig 1

Growth of Dunaliella at various salinities and phosphorus concentrations in ASW.
Dry weight at day 10.

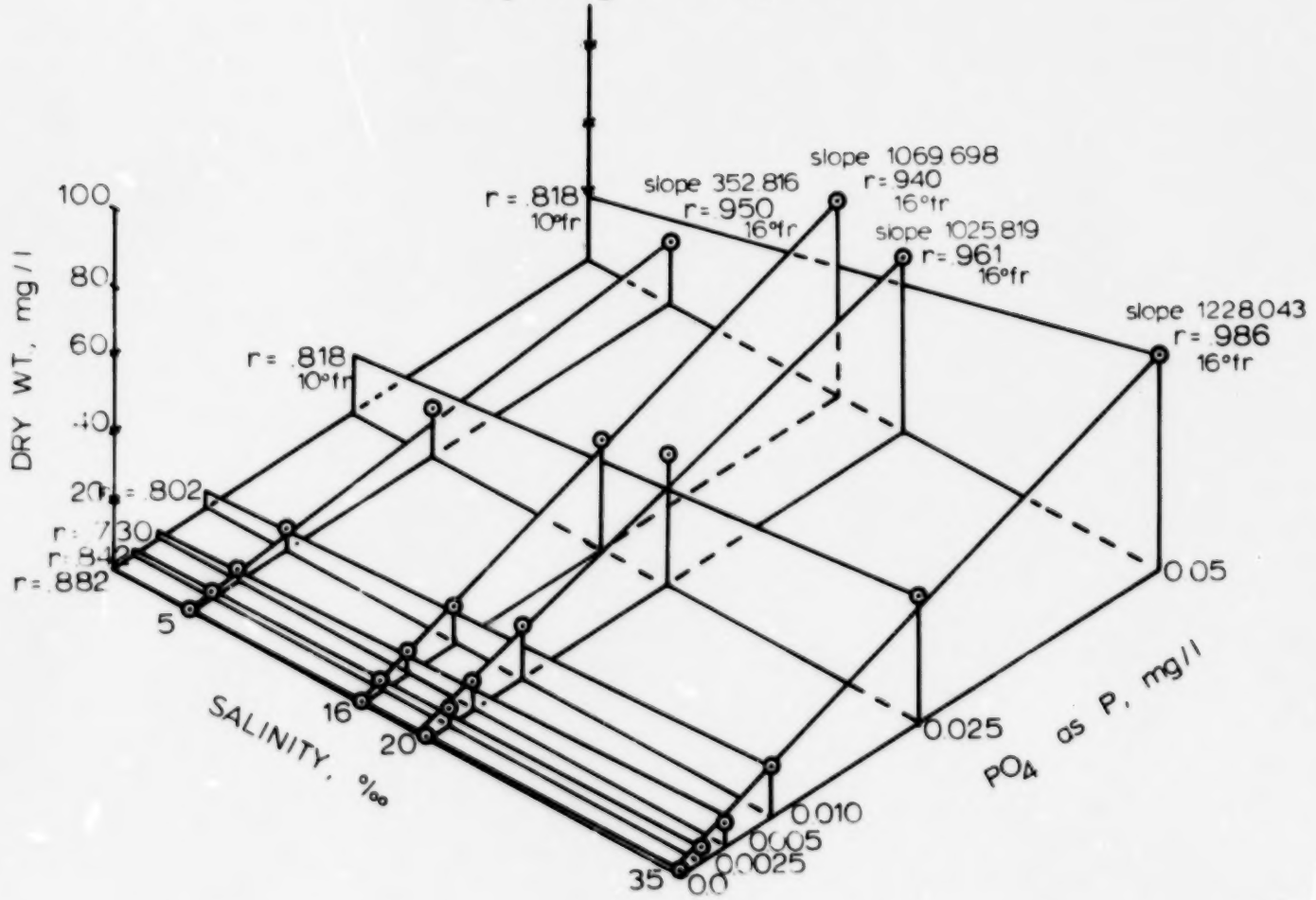


Fig. 2

Growth of Dunaliella at various salinities and nitrate nitrogen concentrations in ASW. Dry weight at day 10.

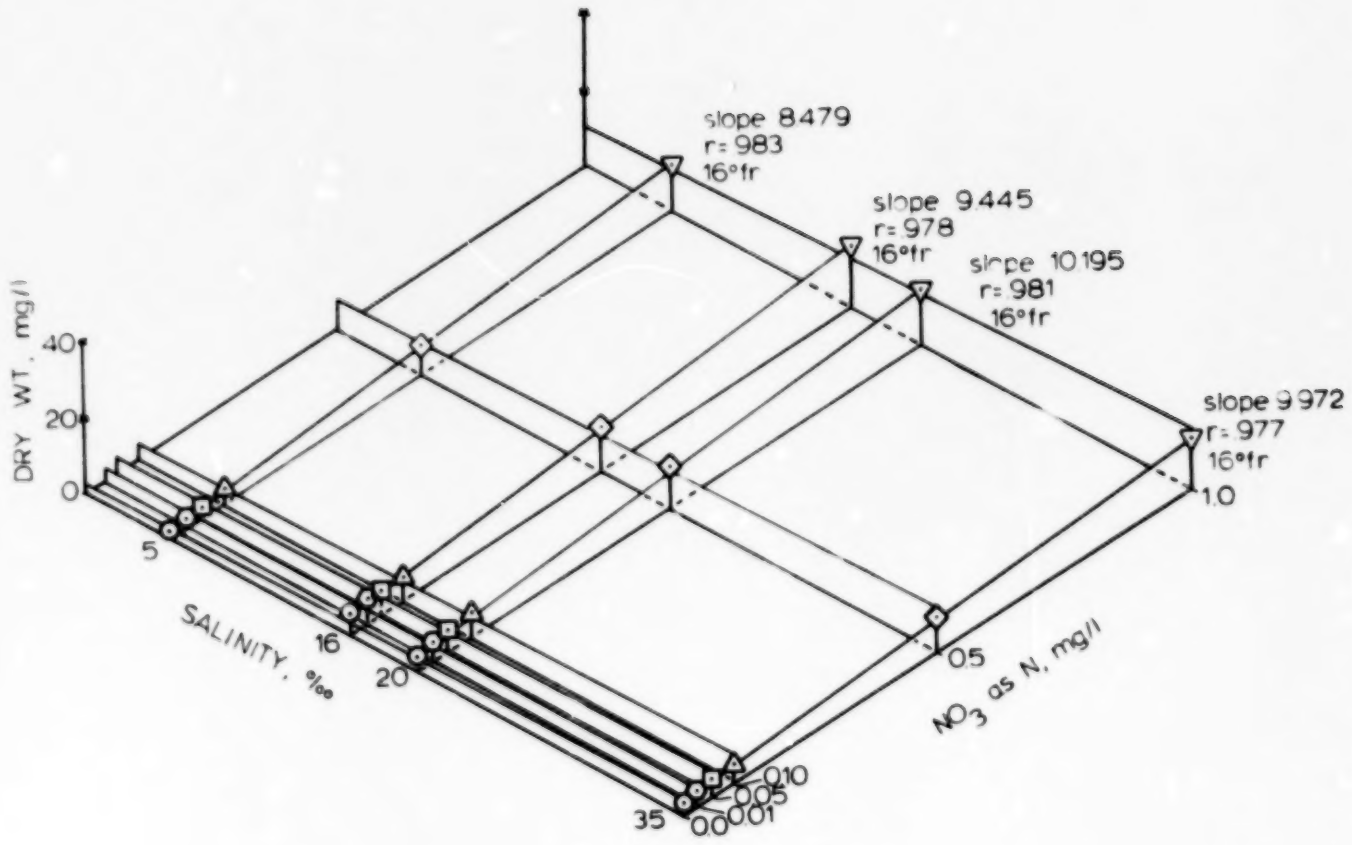


Fig. 3

Growth of Dunaliella at various salinities and ammonia nitrogen concentrations in ASW
Dry weight at day 10

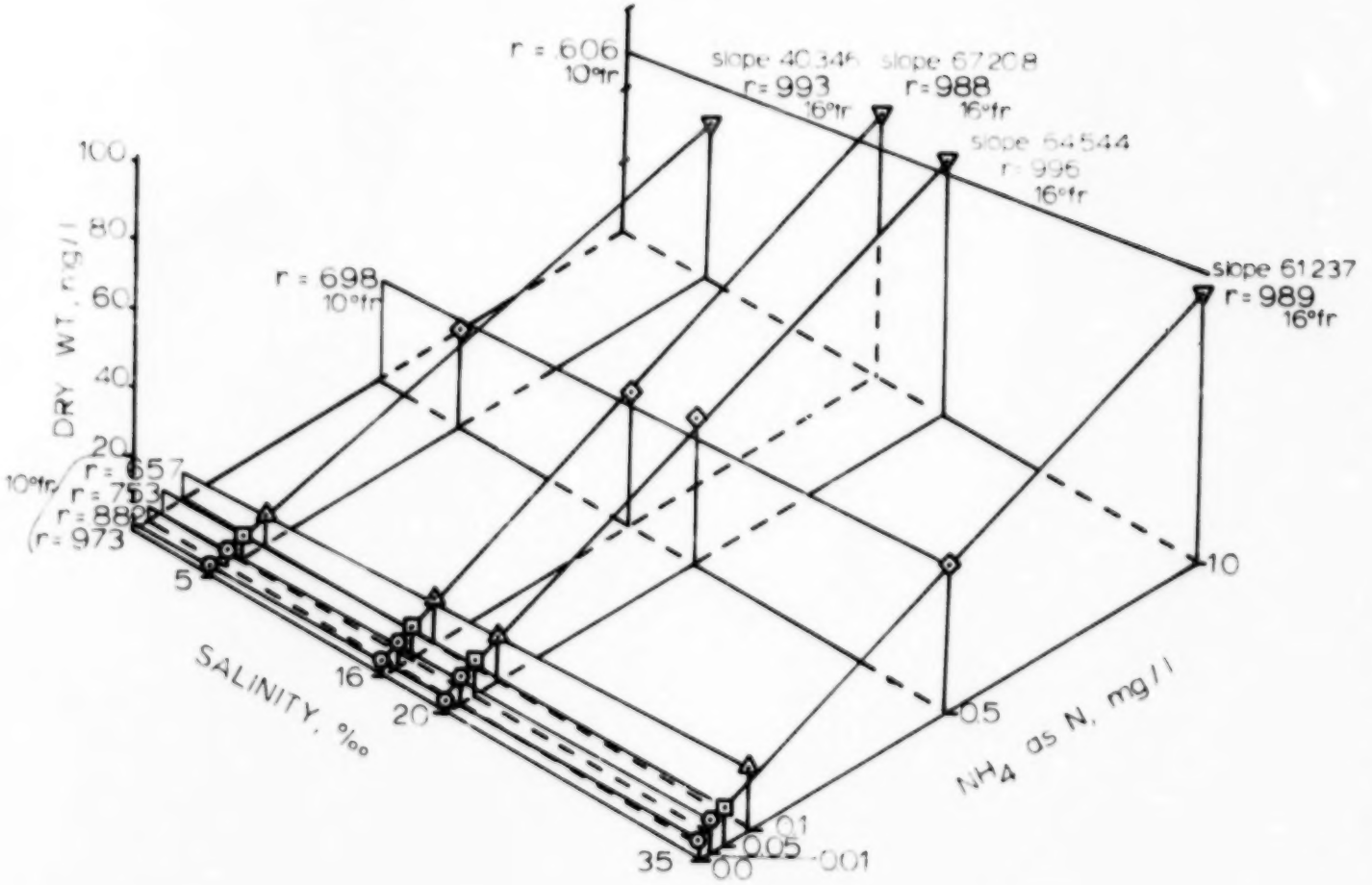
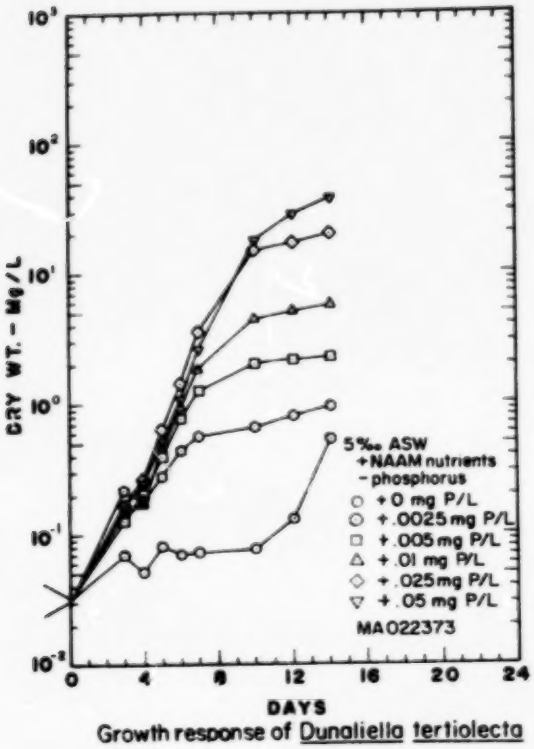
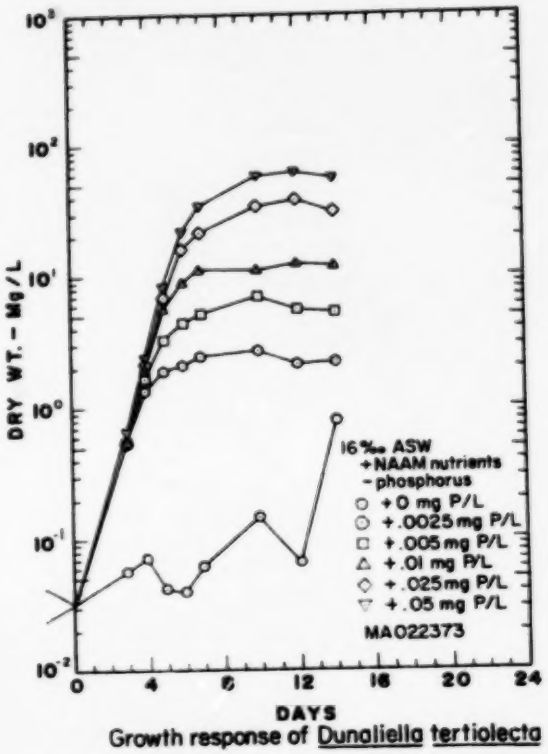


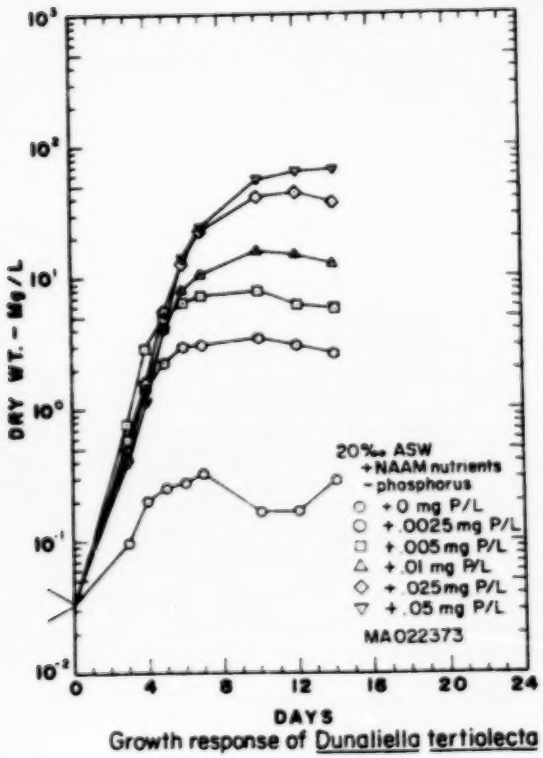
Fig. 4



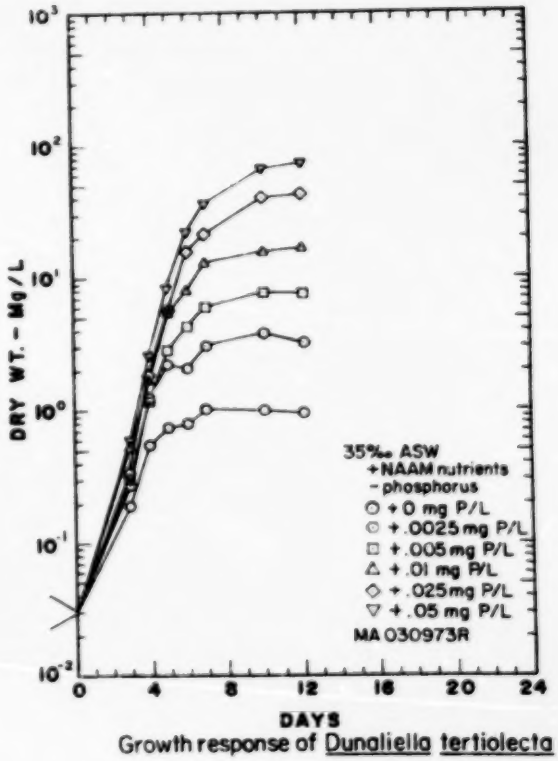
Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta

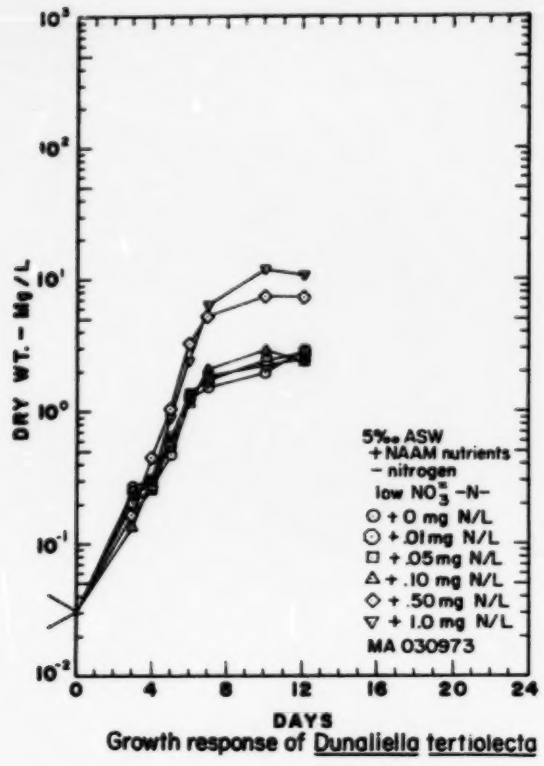


Growth response of Dunaliella tertiolecta

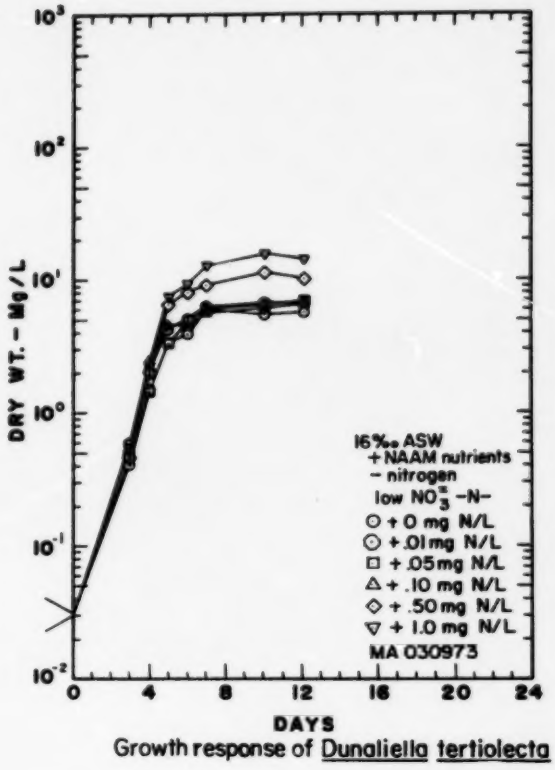


Growth response of Dunaliella tertiolecta

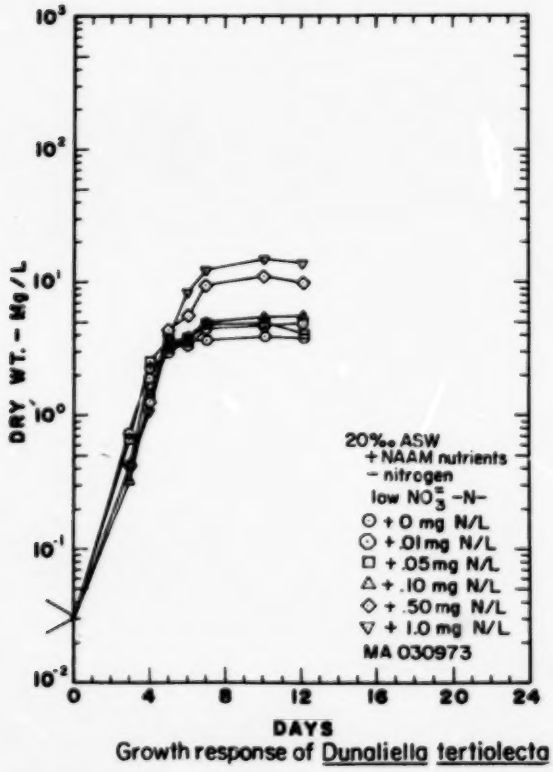
Fig. 5



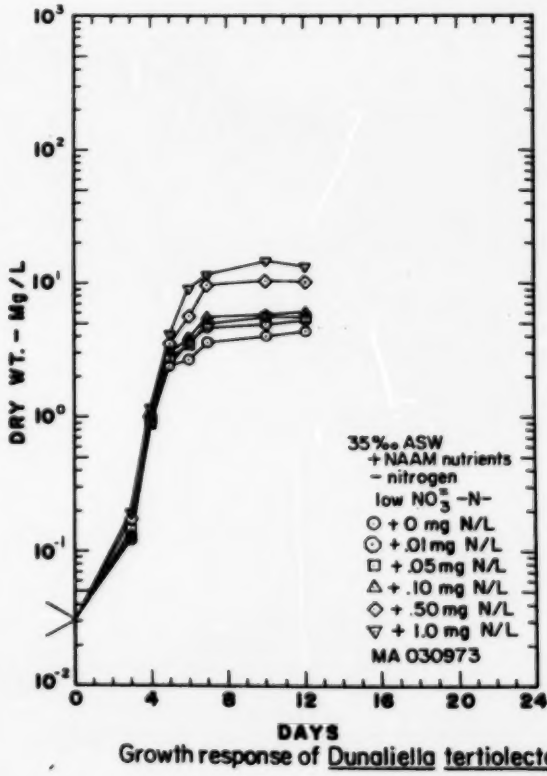
Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta

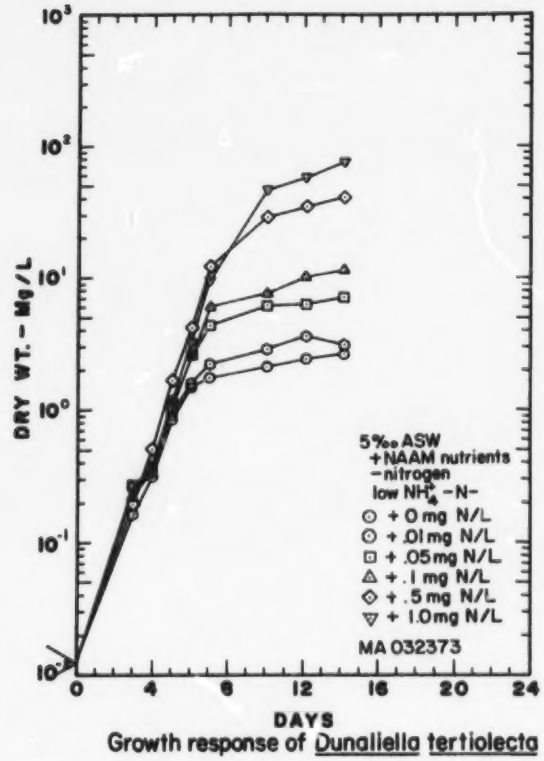


Growth response of Dunaliella tertiolecta

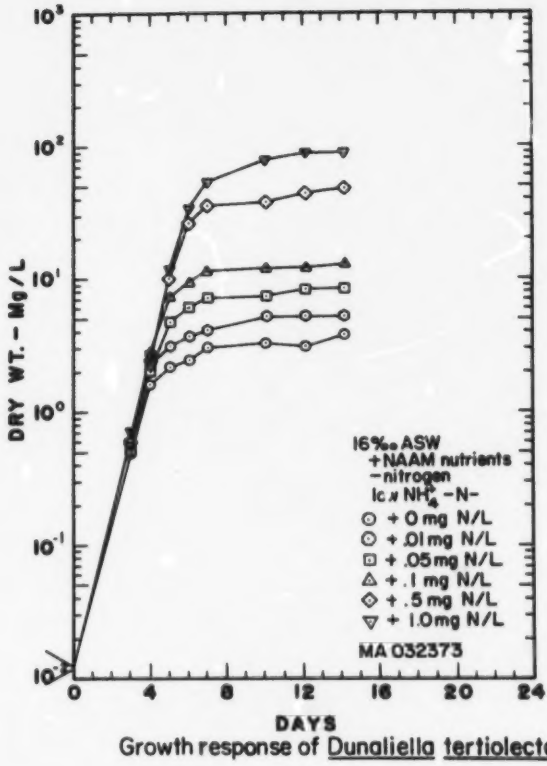


Growth response of Dunaliella tertiolecta

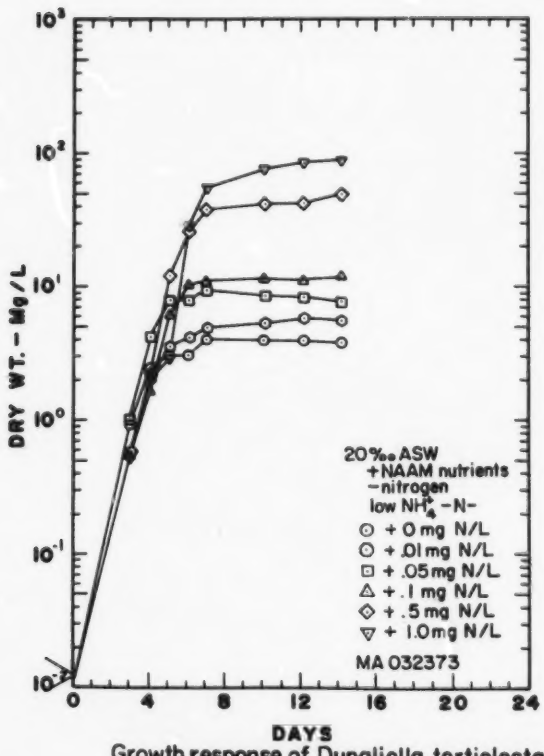
Fig. 6



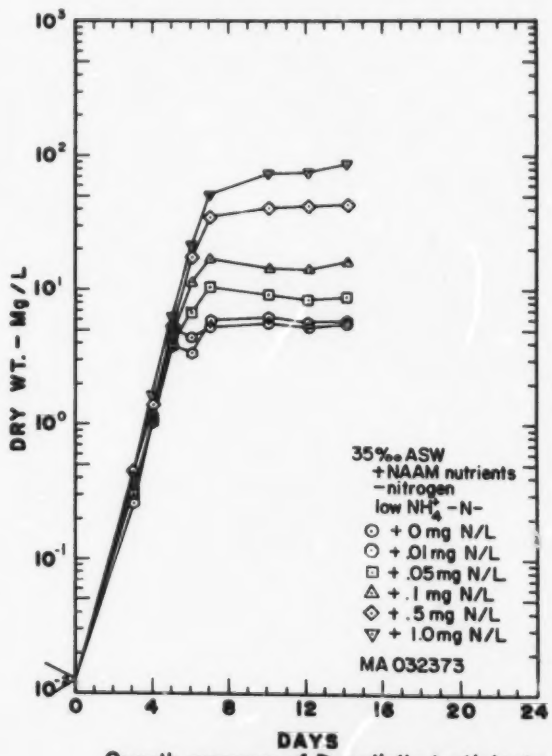
Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta

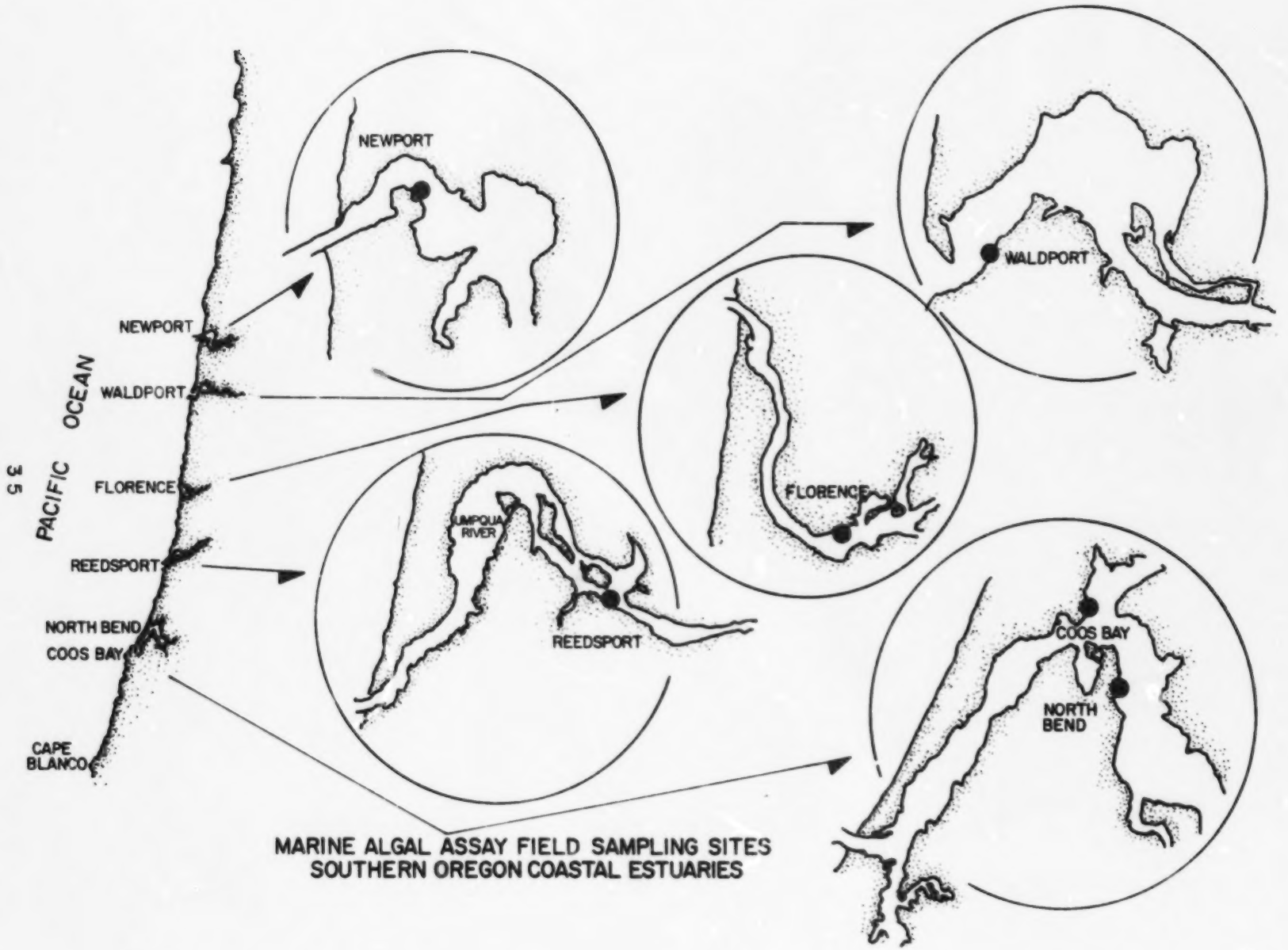
Fig. 7

TABLE 1

BIOMASS PRODUCED PER UNIT OF NUTRIENT BY DUNALIELLA
AT DAY 14 IN DEFINED MEDIA

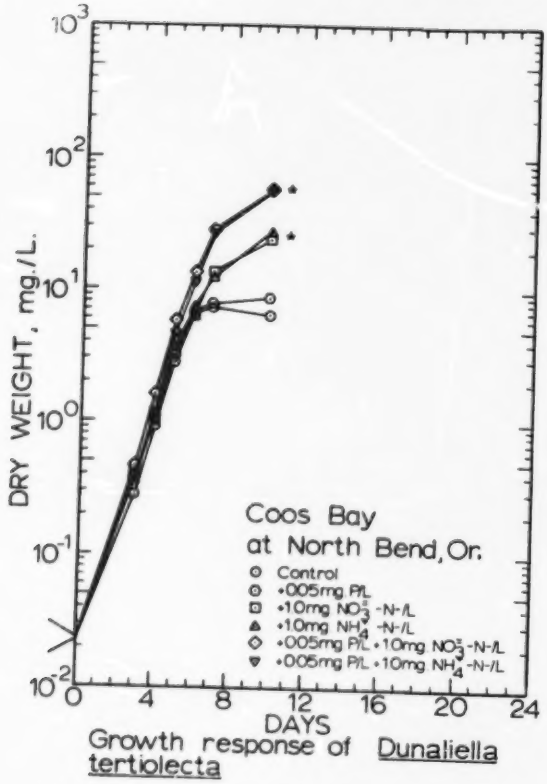
Nutrient / Salinity	mg dry weight/µg of nutrient			P:N ratios	
	P	NO_3^- - N	NH_4^+ - N	P:NO_3^- - N	P:NH_4^+ - N
5 ‰	0.557 ±0.158	0.0096 ±0.0014	0.0747 ±0.0075	1:58	1:7.5
16‰	0.930 ±0.240	0.0308 ±0.0394	0.0844 ±0.0058	1:30.2	1:11.0
20‰	1.170 ±0.164	0.0331 ±0.0379	0.0766 ±0.005	1:35.3	1:15.3
35‰	1.129 ±0.232	0.0315 ±0.0361	0.0765 ±0.0193	1:35.8	1:14.8
Average*	1.076	0.0318	0.0796	1:33.8	1:13.5

*Average is calculated from the 16 ‰, 20‰, and 35‰ data only.

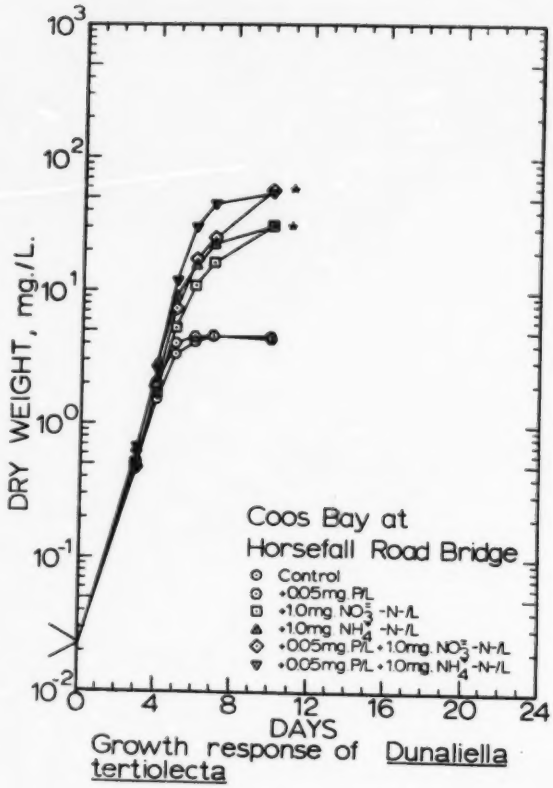


MARINE ALGAL ASSAY FIELD SAMPLING SITES
SOUTHERN OREGON COASTAL ESTUARIES

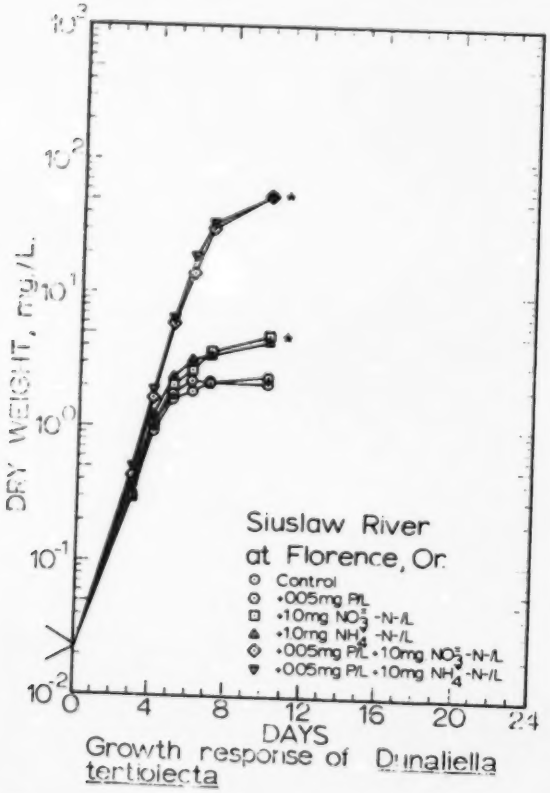
Fig. 8



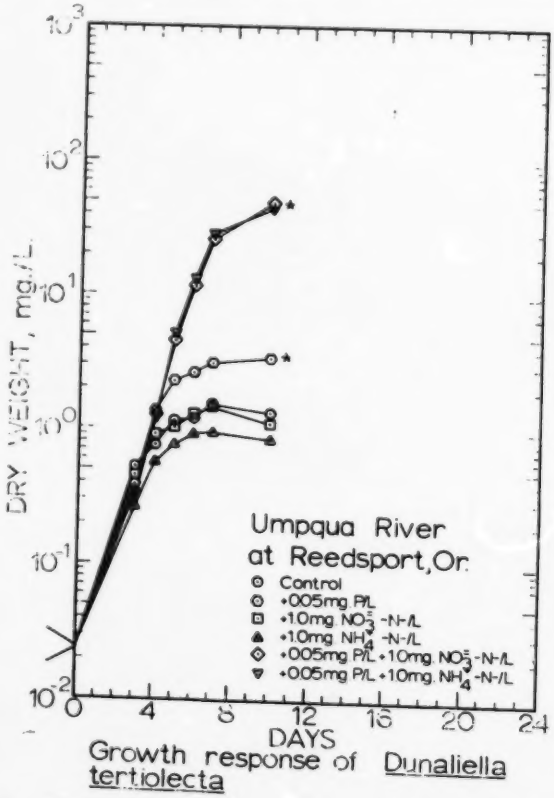
Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta

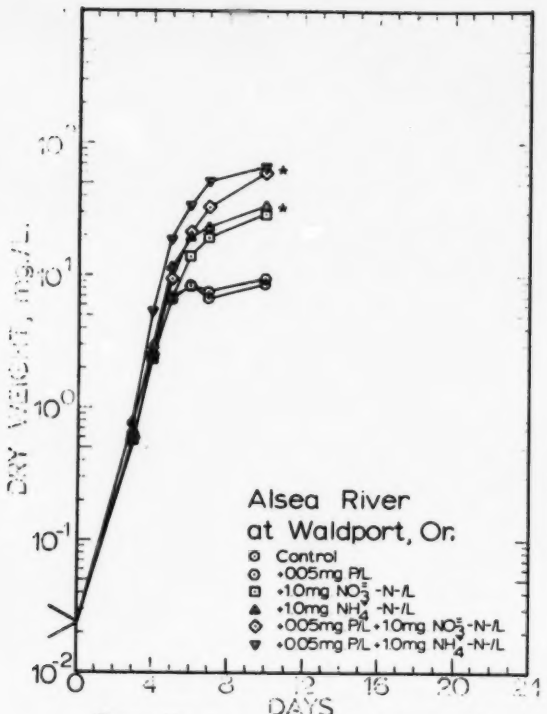


Growth response of Dunaliella tertiolecta

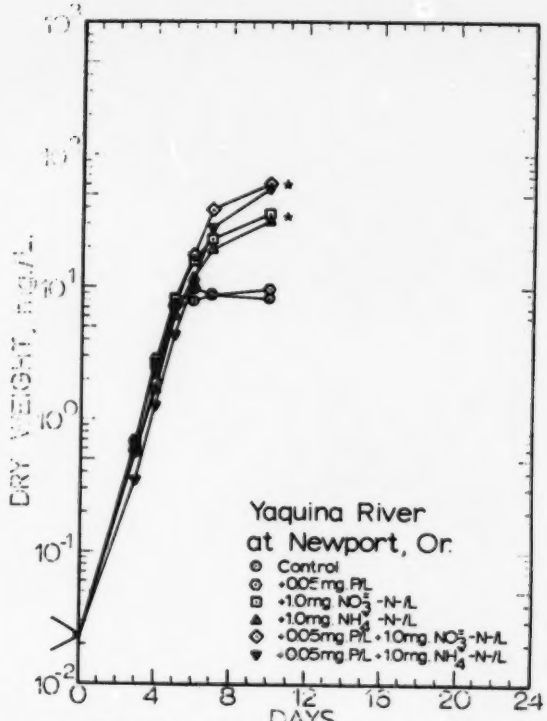


Growth response of Dunaliella tertiolecta

Fig. 9



Growth response of Dunaliella tertiolecta



Growth response of Dunaliella tertiolecta

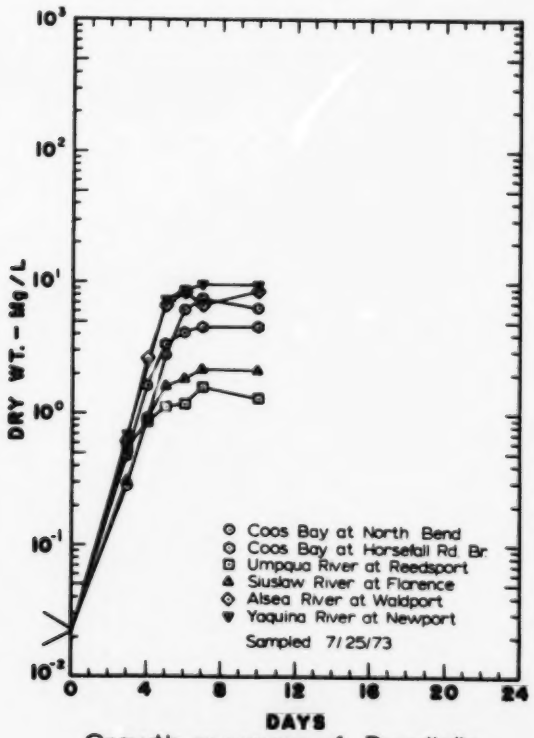


Fig. 10

Growth response of Dunaliella

TABLE 2

YAQUINA BAY, OREGON
Algal assay growth response and associated parameters
from low water-high water samples collected 02/9/'74
(Surface grab samples, membrane filtered)

Sampling Station Treatment	OSU Dock low tide	OSU Dock high tide	Sally's Bend low tide	Sally's Bend high tide	River Bend low tide	River Bend high tide
Control dry wt., day 10, mg/l†	28.9	16.8	21.8	19.2	7.35	27.5
+ 0.05 mg P/l	37.3*	17.1	46.2*	17.3	43.8*	34.7*
+ 1.0 mg N/l	30.5	38.8*	21.6	36.4*	8.1	31.3*
Growth limiting nutrient	P	N	P	N	P	P (N)
Salinity, ‰	19.0	29.0	13.8	28.2	6.7	22.8
Ortho-P	0.024	0.027	0.021	0.027	0.014	0.024
N (NO_2+NO_3+NH_4) mg/l	0.53	0.234	0.685	0.263	0.818	0.478
mg dry wt/µg P	1.20	0.621	1.04	0.710	0.524	1.15
mg dry wt/µg N	0.054	0.071	0.031	0.072	0.008	0.057
mg dry wt/µg of limiting nutrient in ASW (Specht & Miller, in press) adj. for salinity	1.11	0.077	0.810	0.076	0.614	1.16

Linear regression of parameter vs dry wt.	Slope	Intercept	Correlation coefficient (r)	t-test, sign. of (r)	# degrees of freedom
P (of P limited samples)	2090.0	-21.9	0.996*	35.5*	10
N (of N limited samples)	42.1	7.9	0.990*	19.2*	7
Salinity	0.359	13.1	0.392	1.71	16

*indicates statistically significant difference

†all dry weights are geometric means of triplicate samples

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